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Abstract

High levels of arsenic contamination in drinking water pose serious health risks in numerous countries. The documentation reporting arsenic toxicity on reproduction and development is increasing, with evidence of arsenic inducing fertility and developmental issues. Nonetheless, the impact of arsenic exposure on the development of the male reproductive system is not fully elucidated. In the present study, we have investigated the direct effects of arsenic on prepubertal mouse testis using an *in vitro* testicular organ culture system. Culture medium was supplemented with a range of concentrations of sodium arsenite, examining effects of low (0.5 and 1 µM) and high (10, 50, 100 µM) concentrations, in cultures of post-natal day 5 CD1 mouse testis. *In vitro* exposure of low arsenic concentrations (0.5 or 1 µM) for 6 days did not cause any change in the testicular morphology, germ cells density, or apoptotic marker cleaved

caspase 3 (CC3) expression. In contrast, exposure of prepubertal testis to high arsenic concentrations (10, 50 or 100 μM) induced drastic changes: severe destruction of testicular morphology, with loss of seminiferous tubule integrity; a dose-dependent decrease in germ cell density, and a hundred-fold increase in CC3 expression after 50 μM arsenic exposure. In conclusion, high arsenic treatment induced a dose-dependent induction of apoptosis and germ cell loss in prepubertal mouse testis.

Keywords:

Sodium arsenite; *in vitro*; prepubertal mouse; testis; toxicity.

1. Introduction

Arsenic is a metalloid naturally present in food, soil, and water (Duker et al., 2005; Huang et al., 2004) with a toxicity defined by its binding state; inorganic arsenic compounds are more toxic than organic compounds (Maitani et al., 1987; Styblo et al., 2000). Usually, natural water contains inorganic arsenic forms such as trivalent (As III) and pentavalent arsenic (As V) (Feng et al., 2001). Trivalent arsenic, such as sodium arsenite, is more toxic than the pentavalent form, for example sodium arsenate (Styblo et al., 2000; Vega et al., 2001). Arsenic is a potent carcinogen, its chronic exposure can lead to adverse effects on skin and on the cardiovascular, neurological and respiratory systems (reviewed in Abdul et al., 2015; Ratnaike, 2003).

Arsenic poisoning has become an escalating problem in many developing countries. Usually the contamination of drinking waters with arsenic occurs due to natural environmental

sources seeping into aquifers, mining, and other industrial activities (Sulanjari et al., 2015). In particular, in the past few decades its exposure through drinking water has vastly increased in Bangladesh, India, China, and Cambodia (Smith, 2003; Xie, 2011). Recently, the risk of arsenic exposure has also been reported to have worsened in Pakistan, exposing almost 50 million people to high levels of arsenic through drinking groundwater (Podgorski et al., 2017). Overall, more than 150 million people around the world are affected by drinking arsenic-contaminated water (Munoz et al., 2015): its chronic exposure is therefore a major concern for public health (Abhyankar et al., 2012).

There is a clear link between exposure to inorganic arsenic and male reproductive dysfunction (Feng et al., 2001; Kim and Kim, 2015; Lima et al., 2018; Wang et al., 2006). In the testis, exposure to arsenic can alter spermatogenesis, interrupt steroidogenesis, and reduce gonadotrophin as well as testosterone levels (Kim and Kim, 2015). *In vivo* studies investigating the reproductive toxicity of arsenic have shown effects on adult rodent reproductive system. For instance, Chang et al. (2007) reported reduced testicular weight and sperm count in mice after subchronic exposure to 20 or 40 mg/l sodium arsenite. Similarly, mice exposed to sodium arsenite via drinking water at concentration of around 530 μ mol/l presented with a significant reduction in sperm count and motility (Pant et al., 2001). A comparative study in male rats exposed to arsenite or arsenate suggested a higher reproductive toxicity of the inorganic form (Lima et al., 2018). A small number of *in vitro* studies have assessed effects of arsenic on embryonic development and reproduction. Different concentrations of sodium arsenite exert toxic effects on preimplantation development of mouse embryos, with 100 μM arsenic concentration rapidly killing two-cell embryos, while 1 μM impairs blastocyst formation (Muller et al., 1986). *In vitro* studies of arsenic on post-implantation mouse embryo development confirmed that sodium arsenite possesses higher toxicity compared to sodium

arsenate (Chaineau et al., 1990; Tabocova et al., 1996). Moreover, Sertoli cell lines undergo apoptosis after arsenic trioxide exposure (Kim et al., 2011).

In general, children are considered more sensitive to toxicants than adults (Scheuplein et al., 2002). There is an accumulating evidence of a strong negative impact of arsenic exposure in early life, includes link to increased mortality rates during both childhood (Rahman et al., 2013) and also adulthood (Yuan et al., 2007). Prepubertal testicular development is a highly sensitive stage and it is possible that toxicants such as arsenic may act differently on testis of young compared to adult males. Presently, only a few animal studies have examined the effects of arsenic on the reproductive axis of the prepubertal mammal: prepubertal rat prostate development has been found to be affected (Aquino et al., 2019), but that study provides little information about the testicular cell populations that could be damaged (i.e. somatic and/or germ cells); in one other study, prepubertal rats exposed to sodium arsenite (0.01 or 10 mg/l) for 30 days in the drinking water showed a significant disruption of normal testicular morphology (da Cunha de Medeiros et al., 2019).

Here, we have investigated the effect of sodium arsenite exposure on immature mouse testis using an *in vitro* culture system that supports the developing neonatal testis. *In vitro* models have been commonly used to elucidate the metabolism of arsenic (Drobna et al., 2009), and the testis culture model has already been used for other toxicological studies on the male gonad (Allen et al., 2020; Lopes et al., 2016; Nakamura et al., 2019; Smart et al., 2018).

2. Materials and Methods:

2.1. Animals

This work was approved by the Local Ethical Review Committee of the University of Edinburgh and conducted according to UK home office regulations. CD1 male mice, obtained

from University of Edinburgh breeding stock, were kept at 14 h:10 h light and dark photoperiod, provided *ad libitum* food (expanded RM3 diet, SDS; DMB Scotland) and water. All mice used in the experiments were euthanized by decapitation at post-natal day (PND) 5 for tissue collection or by cervical dislocation at PND 12 for age-matched control, with the day of the pup delivery considered as PND 0.

2.2. Tissue culture

PND 5 testes were collected, fragmented and cultured according to Lopes et al. (2016). In brief, for each culture run, testes from at least two mice were dissected, pooled together and placed into dissection medium Leibovitz L-15 (Invitrogen, UK) supplemented with 3mg/ml of bovine serum albumin (BSA; Sigma-Aldrich Ltd, UK) at 37ºC. After removal of epididymis and tunica albuginea, testes were fragmented into approximately 0.5mm^3 pieces, using a scalpel blade. Fragments that were homogeneous in size were randomly distributed on a 24-well culture plate (Greiner Bio-one, UK), with testes obtained from two pups per litter for each culture run. On the first day of culture (Day 1), each testicular fragment was placed on a floating polycarbonate membrane (Whatman, Camlab Ltd, Cambridge, UK) per each well, containing 1 ml α-Minimal Essential Medium (MEM; Invitrogen, UK) supplemented with 10% Knockout Serum Replacement (Invitrogen, UK); half of the medium was replaced with fresh medium on Days 4 and 6. The culture plate was incubated at 34ºC, 5% CO_2. Fragments were left untreated for the first 24 h (Day 1). From Day 2 of culture, testicular fragments were exposed to different concentrations of sodium arsenite prepared from a stock solution of 0.05 mol/l (0.1N) Tritipur®reag. (Merck, Germany). Sodium arsenite solution was diluted in culture medium and final concentrations were prepared through serial dilutions. Two sets of experiments (low and high arsenic doses) were performed with cultures maintained for seven days, of which six days were with arsenic exposure (Days 2-7): the *in vitro* study on mouse embryos arsenic exposure was used as a reference for selection of the highest dose (Muller et al., 1986). The first

experiment (low arsenic doses) comprised of 0.5 and 1 μM sodium arsenite concentrations, whereas the second experiment (high arsenic doses) included concentrations of 10, 50 and 100 μM arsenic. In each culture run, the arsenic effect was compared to a culture control that had been maintained throughout in arsenite-free medium. Each experiment was repeated six times, including six culture runs, six histological and immunohistochemistry (IHC) runs and six sets of image analysis. On Day 7, tissues were fixed for 1 h in 10% neutral buffered formalin (Sigma Aldrich, UK) prior to IHC, or in Bouin's fluid (Sigma Aldrich Ltd, UK) prior to histological examination. Tissues were placed in 3% agar (Sigma Aldrich Ltd, UK) blocks and then embedded in paraffin wax prior to sectioning.

2.3. Histology/ Immunohistochemistry

Five μm thick serial sections were cut. A middle section from each tissue fragment was dewaxed, rehydrated through a series of decreasing alcohol concentrations, stained with hematoxylin and eosin (H&E), and used for morphological analysis. A serial section next to the middle section used for histological examination was selected for IHC reactions. Sections were dewaxed, rehydrated, and processed for antigen retrieval with citrate buffer (10mM sodium citrate, pH 6, Fisher Scientific, UK). In between each step, slides were washed with phosphate-buffered saline (Fisher Scientific, UK) with 0.1% Triton X (PBSTx, Sigma-Aldrich, UK). Blocking used 20% normal goat serum diluted in PBSTx supplemented with 5% w/v BSA for 1 h at room temperature. Primary antibodies were applied and incubated overnight in a humidified environment: (i) rabbit anti-cleaved caspase 3 (CC3) antibody (9661S, 1:500 dilution; Cell Signaling Technology, USA) and (ii) mouse anti-DEAD-box helicase 4 (DDX4)/Mouse Vasa Homologue (MVH) antibody (ab27591, 1:100 dilution; Abcam, UK). After washing, appropriate secondary antibodies were applied (both 1:200 dilution): (i) goat anti-rabbit biotinylated (E0432, DakoCytomation, Denmark) and (ii) Alexafluor goat anti-mouse IgG1 568nm (A-21124, Invitrogen, UK). Omission of primary or secondary antibodies

were used as technical negative controls. For CC3, the visualization reagent Streptavidin Alexa Fluor 488 conjugate (S-32354, Invitrogen, UK) was subsequently applied at 1:200 dilution. All sections were counterstained with 4,6-Diamidino-2-phenylindole (DAPI; Invitrogen, UK) and mounted with Vectashield hard-set mounting medium (Vector Laboratories, USA).

2.4. Image attainment and analysis

H&E stained sections were photomicrographed (DMLB Leica microscope, Leica Microsystems Ltd, UK), with images taken using a DFC369FX camera on a Leica DM5500B microscope (Leica Microsystem Ltd, UK), using filters for DAPI, A4 (BP 360/40); for Alexa Fluor 488, N3 (BP 546/12); for Alexa Fluor 568, Y5 (BP 620/60). Image J software was used for image analysis, with the assessor blind as to treatment. Germ cells (MVH^+) were analyzed by manual counting and the total number given relative to the area of seminiferous tubule, in order to obtain a value for germ cell density (number of cells that were MVH-positive divided by seminiferous tubule area in mm^2). Density was also determined for whole section area (number of MVH positive cells divided by the area of section in mm^2) to allow determination of germ cell density even where tubule integrity had been disrupted to the extent that precluded measurement of tubule area. For CC3, fluorophore area was measured as a percentage of tubule, interstitium and section area (proportion of CC3-positive area in mm^2 relative to area of seminiferous tubule, interstitium or whole section, all in mm^2).

2.5. Statistical analysis

GraphPad Prism was used for statistical analyses. The statistically significance of any difference between the control group and the arsenic-treated groups was determined by one-way ANOVA, followed by Dunnett's *post hoc* test where the ANOVA was statistically significant. For analyses including only 2 experimental groups (comparison of the control group to the 10 μM sodium arsenite treatment group for: density of germ cells in the

seminiferous tubules, and percentage of seminiferous tubule or interstitium area positive for CC3), paired T-test was performed. Data is expressed as mean ± SEM, with $p \leq 0.05$ considered statistically significant.

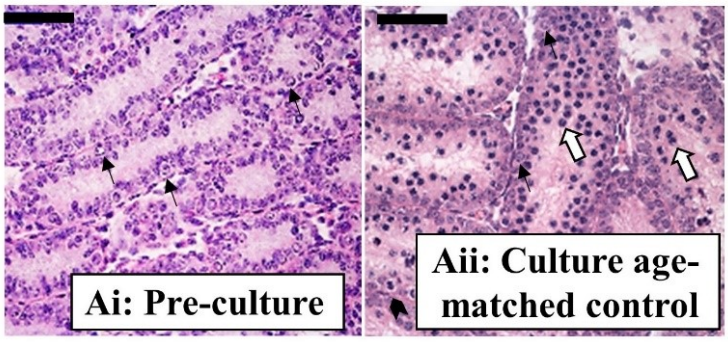
3. Results

3.1. Morphological analysis

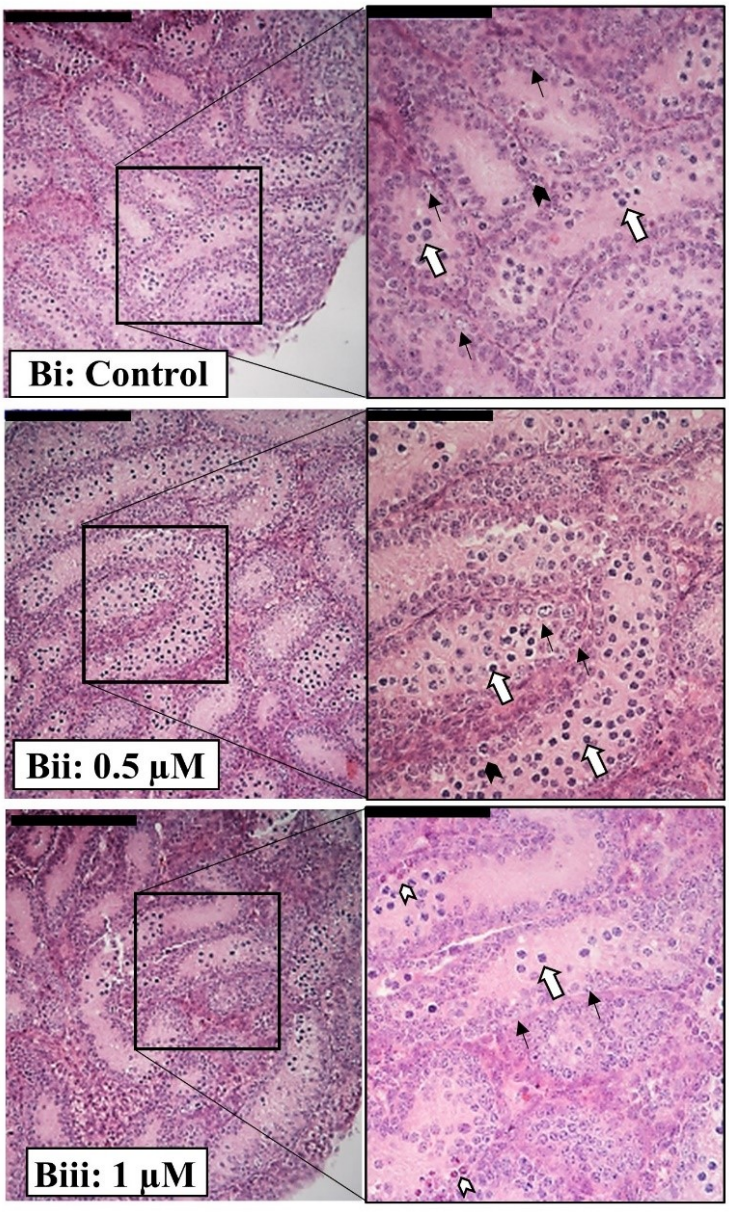
To assess the impact of sodium arsenite on the developing testis of prepubertal (PND 5) mouse, testis fragments were exposed *in vitro* for six days to low or high concentrations of sodium arsenite, compared with arsenite-free culture control testicular fragments in each case. Morphological changes were evaluated on H&E stained sections (Fig. 1).

Figure 1

A: Uncultured control



B: Low Sodium Arsenite Doses Experiment



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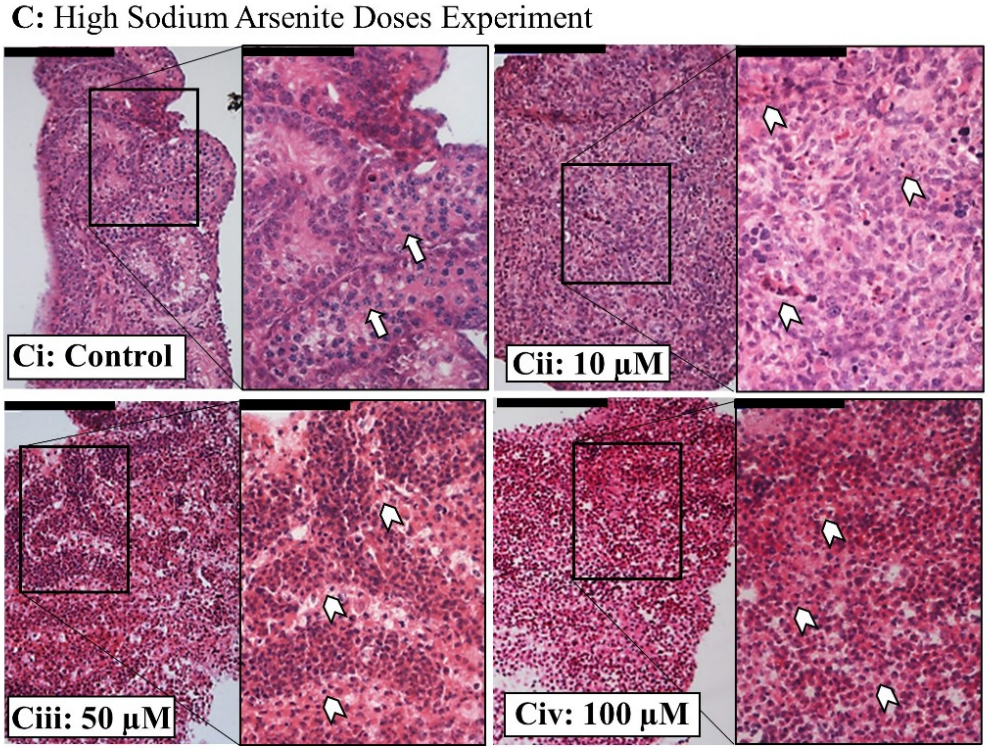


Figure 1. Morphological changes of prepubertal mouse testis induced by *in vitro* exposure to sodium arsenite. Representative images of hematoxylin and eosin stained sections of uncultured and cultured testis fragments. Pre-culture control tissue (**Ai**) obtained from PND 5 mouse showed germ cells only at the spermatogonial stage (**black arrow**), while testes from mice age-matched to be equivalent to end of culture control testis (PND 12: **Aii**) showed germ cells that have entered meiosis (spermatocytes, **white arrow**). Representative images showing the effect on testis morphology of low (**B**) and high (**C**) concentrations of arsenic. Control tissues in both the low dose (**Bi**) and high dose experiment (**Ci**), as well as low doses of arsenic (**Bii**: 0.5 µM sodium arsenite; **Biii**: 1 µM sodium arsenite) preserved normal morphology, with germ cells lining the basal membrane (**black arrow**), some cells undergoing mitosis (**black arrow head**) and several differentiating germ cells (spermatocytes, **white arrow**) accumulating in the forming lumen of well-defined seminiferous tubules. High doses of arsenic caused dramatic morphological changes, starting at 10 µM sodium arsenite (**Cii**) with evidence

of apoptotic/necrotic cells (**white arrowhead**), and with disruption or loss of seminiferous tubule integrity at 50 μM (**Ciii**) and 100 μM sodium arsenite (**Civ**). B-C: scale bars represent 100 μm; A and insets: scale bars represent 50 μm.

The morphology of control testicular fragments was well preserved at the end of the six day culture period for both sets of experiments: necrosis was not present in the centre of the section, seminiferous tubule structure and integrity was maintained, with many germ cells lined along the basement membrane of all tubules, several actively undergoing a mitotic division (Fig. 1 Bi and Ci). Control tissues closely resembled the morphology of equivalent age-matched PND 12 testis (Fig. 1 Aii), with mitotically active spermatogonia lining the basal lamina and spermatocytes in the lumen of the seminiferous tubules, showing that the culture system supports physiological testicular development, given that pre-culture control testis displayed presence of germ cells only at the spermatogonial stage (Fig. 1 Ai). Exposure to low sodium arsenite concentrations did not cause any morphological changes in seminiferous tubules when compared to the control group: seminiferous tubules maintained their physiological shape with abundant spermatogonial germ cells present in tissues treated with both low arsenic doses (0.5 or 1 μM; Fig. 1 Bii and Biii). In contrast, higher concentrations of sodium arsenite severely disrupted normal testicular morphology (Fig. 1 Cii, Ciii, and Civ). Apoptotic/necrotic cells were present, as evidenced by dense dark staining of the chromatin and more intense pink/red staining of the cytoplasm, and seminiferous tubules were irregularly defined, particularly at the 50 or 100 μM arsenic doses.

3.2. Germ cell density

Density of spermatogonial germ cells (MVH^+) was assessed at the end of the culture period for both sets of experiments, low and high arsenic doses (Figs 2 and 3 respectively).

Figure 2

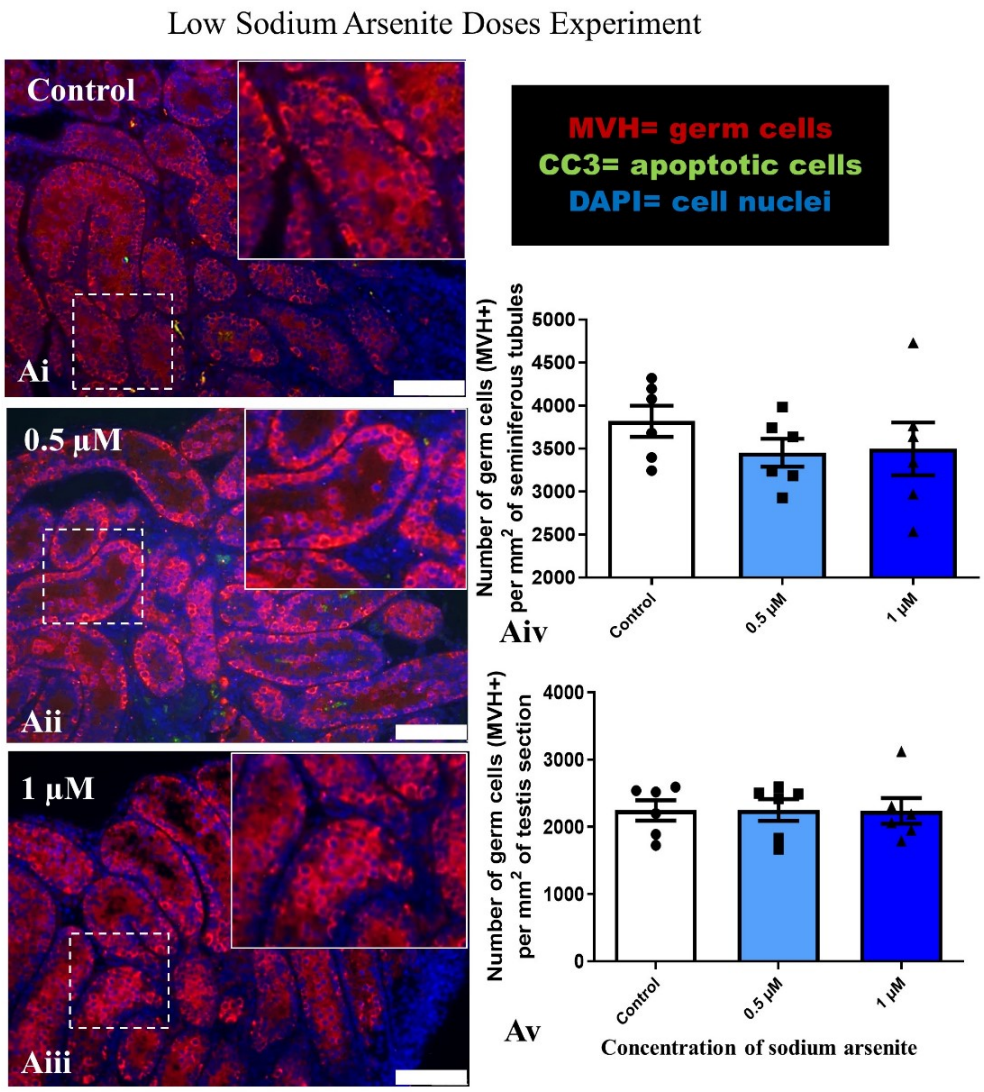
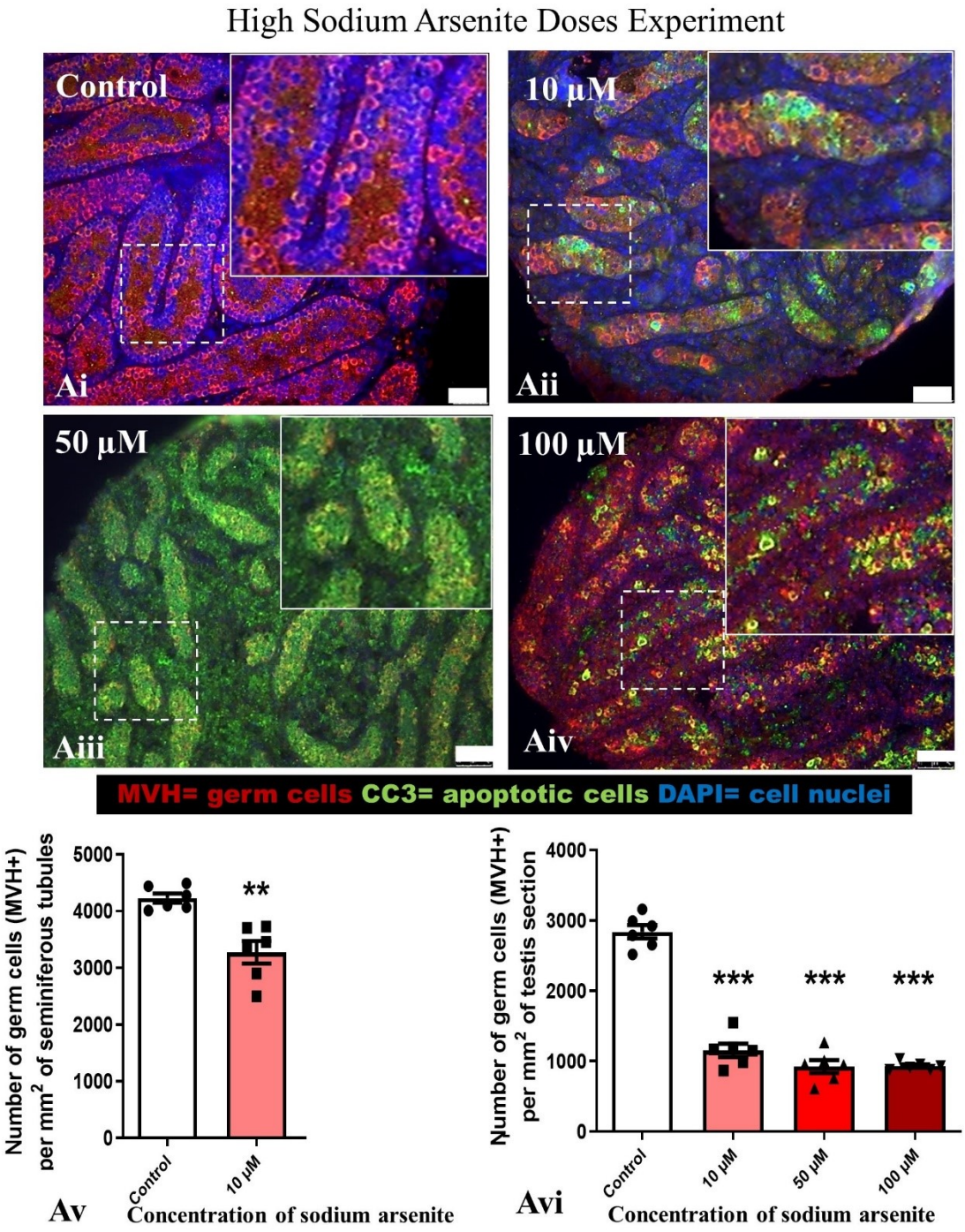


Figure 2. Effect of low sodium arsenite concentrations *in vitro* exposure on germ cell density and apoptosis in the prepubertal mouse testis. Photomicrographs showing immunohistochemical localization of MVH (red) and CC3 (green) expression, counterstained with DAPI (blue) in representative images of low dose arsenic experiment. In control tissue (**Ai**), as well as in the testis fragments exposed to low doses of arsenic (**Aii**, 0.5 µM sodium arsenite; **Aiii**, 1 µM sodium arsenite), germ (MVH^+) cells were abundant and apoptotic ($CC3^+$)

areas were sparse. Scale bars represent 100 μm. Graphs show germ cell density: the number of MVH^+ cells per mm^2 of seminiferous tubules (**Aiv**) and per mm^2 of section (**Av**). Density of germ cells was unaffected in the low arsenic dose treated testis fragments compared to that of control tissue. Data represents individual data points (N=6), mean ± SEM.

Figure 3



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236 **Figure 3. Effect of high sodium arsenite concentrations *in vitro* exposure on germ cell**
237 **density and apoptosis in the prepubertal mouse testis.** Photomicrographs showing
238 immunohistochemical localization of MVH (red) and CC3 (green) expression, counterstained
239 with DAPI (blue) in representative images of high dose arsenic experiment. In control tissue

(**Ai**), germ (MVH^+) cells were abundant, and apoptotic ($CC3^+$) areas were sparse. In contrast, in all arsenic treated groups (**Aii**, 10 μM; **Aiii**, 50 μM; **Aiv**, 100 μM sodium arsenite) a lower density of germ cells was present, with vast areas of CC3 expression. Graphs show germ cell density: the number of MVH^+ cells per mm^2 of seminiferous tubules (**Av**) and per mm^2 of section (**Avi**). A significant reduction in germ cell density occurred in all three groups exposed to high concentrations of sodium arsenite compared to control tissue. Note that it was not possible to obtain data about germ cell density within seminiferous tubules in the 50 and 100 μM arsenic dose groups due to severe loss of tubule integrity. Data represent individual data points (N=6), mean ± SEM. ** and *** indicate $p < 0.01$ and 0.001, respectively.

Control tissues had many MVH^+ cells lining the seminiferous tubule basal membrane (Fig. 2 Ai and 3 Ai). Similarly, germ cells were numerous in testis fragments exposed to low doses of sodium arsenite (Fig. 2 Aii and Aiii). In contrast, testis exposed to high doses of arsenic contained only sparsely distributed germ cells (Fig. 3 Aii, Aiii, and Aiv). Analysis confirmed that, in comparison to control tissue, germ cell density per tubule area and per section area of treated tissues in the low arsenic dose experiment remained unaffected (p-value 0.47 and 0.99 for 0.5 and 1 μM respectively; Fig. 2 Aiv and Avi); in contrast, in the high arsenic doses experiment, sodium arsenite exposure caused a significant decrease in germ cell density (Fig. 3 Av and Avi). Compared to the control group, germ cell density per tubule area was significantly reduced in 10 μM arsenic treated tissues, even though tubule integrity was still maintained ($p < 0.01$) (Fig. 3 Av). Due to seminiferous tubule disruption in tissues exposed to higher doses of sodium arsenite, it was not possible to accurately define tubule margins: as a consequence, in these cases density of MVH^+ cells was assessed only relative to testis section area (Fig. 3 Avi): germ cell density per area of testis section decreased by a 2.5-fold magnitude

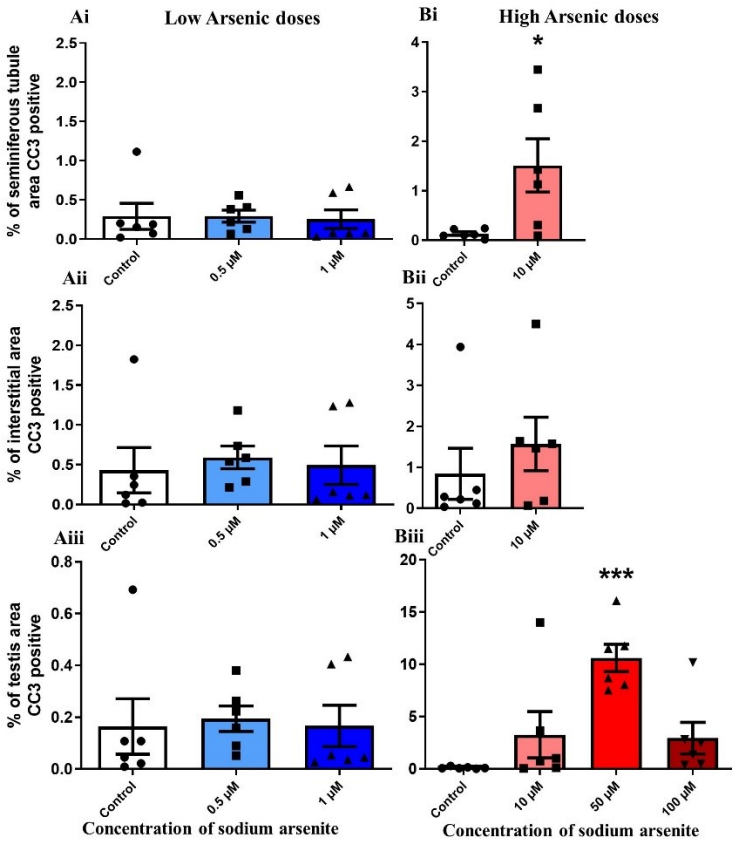
after 10 μM arsenic treatment, while after 50 or 100 μM exposure the decrease was 3-fold for all three doses ($p < 0.001$ for all).

3.2. Analysis of apoptosis

Activation of the apoptotic marker CC3 was measured at the end of the culture period. In the experiment using low arsenic doses, expression of CC3 was low overall, with arsenic exposed tissues (Fig. 2 Aii and Aiii) similar to those of control tissues (Fig. 2 Ai). In contrast, exposure to high doses of sodium arsenite induced increased CC3 expression (Fig. 3 Aii, Aiii, and Aiv) compared to control tissue (Fig. 3 Ai).

Analysis of apoptosis after exposure to low arsenic doses, revealed that the treatment did not affect the area of CC3 expression (Fig. 4 A): the percentage of the seminiferous tubule (Fig 4 Ai), interstitium (Fig 4 Aii) and total section (Fig 4 Aiii) area expressing CC3 was similar to that of control tissue ($p = 0.968$, 0.88 and 0.958, respectively).

277 **Figure 4**



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280 **Figure 4. Apoptosis of the prepubertal mouse testis was induced by *in vitro* exposure to**
281 **high arsenic doses.** Percentage of CC3 positive area of testicular fragments was not affected
282 by low arsenic doses exposure compared to control (**Ai**, **Aii**, **Aiii**). However, exposure to 10
283 µM arsenic increased the CC3 positive area of seminiferous tubules (**Bi**). In the interstitium,
284 exposure to 10 µM did not affect CC3 expression compared to control tissue (**Bii**). The
285 percentage of CC3 expression per total section area was increased compared with control after
286 50 µM arsenic exposure only (**Biii**). Note that it was not possible to obtain data about apoptosis
287 within seminiferous tubules in the 50 and 100 µM arsenic dose groups due to the severe loss
288 of tubule integrity. Data represent individual data points (N=6), mean ± SEM. * and ***
289 indicate $p < 0.05$ and 0.001, respectively.

As per the analysis of germ cell density, in the high arsenic dose experiment, the loss of the seminiferous tubule integrity after exposure to 50 and 100 μM sodium arsenite rendered it impossible to analyze CC3 expression per unit tubule area. In the 10 μM treatment group, 1.5% of the seminiferous tubule area expressed CC3, significantly increased over expression in the control tissue ($p < 0.05$; Fig. 4 Bi). No change was seen outside of the tubule areas, with the percentage of the interstitium expressing CC3 unaffected by treatment ($p = 0.43$; Fig. 4 Bii). When examining the tissue as a whole, CC3 expression increased a hundred-fold in tissues exposed to 50 μM sodium arsenite compared to control tissue (Fig. 4 Biii). All three of the high arsenic doses exhibited increased CC3 expression compared to control tissue (3.2%, 10.5%, and 2.9% of the section area CC3-positive in the 10, 50, and 100 μM treatment groups respectively, compared to 0.1% of the tissue CC3-positive in the control tissue), although only the 50 μM sodium arsenite group reached statistical significance ($p < 0.001$; Fig. 4 Biii).

4. Discussion

Arsenic is a well-known carcinogen present naturally in the Earth's crust. Human and animal exposure to arsenic is a common consequence of consumption of contaminated food or water, or arising from the use of contaminated water for crop irrigation. Arsenic is also used in a variety of industrial processes, contributing to its environmental distribution as well as representing a risk of occupational exposure. Both chronic and acute exposure to arsenic can contribute to a spectrum of diseases in humans. Effects of arsenic on adult male reproduction has been studied, with reports that arsenic inhibits steroidogenesis, causes spermatotoxicity, and adversely impacts on the weight of the reproductive organs of mice *in vivo* (Pant et al., 2004; Sarkar et al., 2003). Similarly, *in vivo* arsenic exposure induces biochemical and

morphological changes in adult rat testis (de Araujo Ramos et al., 2017). Given the marked differences between the pre- and post-pubertal testis, it is important to investigate effects of sodium arsenite directly on the developing male reproductive tissues; with, in contrast, little information available: thirty-day administration of sodium arsenite to prepubertal rats induced dysfunction of normal prostate development (Aquino et al., 2019), spermatogenesis and epididymal structure (da Cunha de Medeiros et al., 2019). Here, we have used an *in vitro* culture system of prepubertal mouse testis to assess the impact of arsenic on the testicular development. The culture system used is capable of supporting testicular development (Sato et al., 2011) and has already been used by our group (Allen et al., 2020; Lopes et al., 2016; Smart et al., 2018) as well as others (Chapin et al., 2016; Nakamura et al., 2019; Park et al., 2020) for toxicological studies on the prepubertal testis. In the results reported here, consistency and health of the control tissue, along with the presence of active spermatogenesis within the vast majority of seminiferous tubules and the absence of any necrotic central area, strengthens the suitability of this culture model for the evaluation of the short-term impact of a toxicant on the immature testis (Chapin et al., 2016; Nakamura et al., 2019). Use of such *in vitro* models, in addition to allowing strict control over exposure conditions, also complies with regulations requiring reduction of animal suffering by avoiding *in vivo* administration. This is particularly important here because there is little information about the toxicological effect of arsenic upon neonatal mice and because it allows examination of the effects of arsenic on the early reproductive system whilst avoiding the inherent difficulties that come with administration of a toxicant to early neonatal animals (Twaddle et al., 2019). Nonetheless, the absence of physiological pharmacokinetics of arsenic in the body, inevitably influencing circulating and tissue arsenic levels, needs to be taken into account when extrapolating data from the present work, as for any *in vitro* study. It is known that some metabolic processes, such as methylation, increase renal clearance, reducing arsenic toxicity, although it can also produce even more cytotoxic

compounds (Drobna et al., 2009; Twaddle et al., 2018a). Furthermore, a non-linear relationship has been observed between dose of arsenic administered to experimental animals and its metabolism and toxicity, given which it could be difficult to interpret *in vivo* experiments that use doses higher than human exposure (Twaddle et al., 2018b).

Exposure to low sodium arsenite concentrations (0.5 or 1 μM) for six days did not appear to affect mouse testis integrity, with regularly shaped seminiferous tubules lined by germ cells at spermatogonial and spermatocyte phases. On the other hand, high arsenic concentrations (10, 50, or 100 μM) caused significant changes in the testicular structure, with a dose-response pattern: sodium arsenite at 10 μM concentration induced a significant damage with a number of degenerating cells, despite leaving the seminiferous tubule morphology reasonably well distinguishable, while both 50 and 100 μM sodium arsenite treatments drastically compromised seminiferous tubule structure. In all three high arsenic doses, the germ cell population was drastically reduced. The extensive disorganization along with loss of germ cells, shape, as well as shrinkage of the seminiferous tubule observed in the present study after high arsenic exposure is in line with similar observations previously reported in the adult testis after arsenic exposure *in vivo* (Baltaci et al., 2016; Ferreira et al., 2012). A study on the effect of arsenic on prepubertal rats showed abnormal testicular structure with impairment of Sertoli cell number (da Cunha de Medeiros et al., 2019). That study revealed no dose-response pattern, with the both low and high doses administered causing similar effect on the testis, in contrast with results here where a difference effects are observed in low and high concentrations. Nonetheless, a direct comparison between the two studies is challenging, as the *in vivo* study on rats used only two arsenic doses and for an extended (30-day) period of exposure (da Cunha de Medeiros et al., 2019), while our *in vitro* study exposed mouse tissues to five different concentrations for six days. A dose-dependent reduction of germ cells has been already

363 reported in adult mice exposed *in vivo* to sodium arsenite both short- and long-term
364 (Sanghamitra et al., 2008; Zeng et al., 2019).

365 Results here also showed an increase in the density of apoptotic cells present within
366 seminiferous tubules after exposure to 10 μM sodium arsenite, with an increase in apoptosis
367 also seen in the 50 μM sodium arsenite treatment group: in the latter case, tubules were ill
368 defined, and so analysis could only examine density of apoptotic cells across the entire
369 testicular section. Paradoxically, the increased expression of CC3 after exposure to 100 μM of
370 sodium arsenite did not reach statistical significance. One possible explanation could be that at
371 the top dose, apoptotic cells died shortly after arsenic administration and the CC3 signal had
372 disappeared relatively rapidly, long before the six days culture end point: the entire apoptotic
373 cell death process can be complete within as short a time as 2-3 hours (Elmore, 2007).
374 Apoptosis is self-destruction process which can occurs by intrinsic and/or extrinsic pathways
375 (Dua et al., 2015). Reactive oxygen species (ROS) produced by mitochondria are considered
376 as the biochemical mediators of apoptosis (reviewed in Orrenius et al., 2015), thus a high
377 enough accumulation of ROS can initiate apoptosis (reviewed in Redza-Dutordoir and Averill-
378 Bates, 2016). Arsenic is known to cause oxidative stress by increasing ROS production and
379 reducing antioxidant defense systems (Yamanaka et al., 1991). Available literature indicates
380 that *in vivo* arsenic exposure can cause an increase in testicular oxidative stress and apoptosis
381 (Das et al., 2009; Uygur et al., 2016). Here, it is possible that high arsenic exposure to the
382 prepubertal testis culture might have induced ROS generation and oxidative stress, thus
383 resulting in cleaved caspase-3-mediated apoptosis.

384 According to the World Health Organization (WHO), water is safe for drinking only
385 where there are concentrations of arsenic that are less than 10 parts per billion (ppb) (Brown
386 and Ross, 2002), but the concentrations of arsenic in the drinking water in some areas of
387 developing countries such as Pakistan, Bangladesh, Bihar and India are well above that limit

388 (Brammer and Ravenscroft, 2009; Sanjrani et al., 2017). For instance, in Bangladesh, the
389 national standard for arsenic in drinking water is set at 50 ppb, although survey results have
390 shown that 25% of wells providing domestic water exceed even that level (Ravenscroft, 2005).
391 Analysis of water from irrigation shallow tube wells in Bangladesh has revealed that most of
392 them produce water with levels above 100 ppb arsenic, in several instances even above 200
393 ppb arsenic (Islam, 2005). Top arsenic concentrations of 1891 ppb have been found in Bihar
394 and India (Ghosh, 2007), with the highest of all in Nepal, 2629 ppb arsenic (Shresta, 2003).
395 The doses of sodium arsenite used in the present study are greater than the WHO safe limits of
396 arsenic in the drinking water, but the doses used in the low arsenic study are well within the
397 range of arsenic concentrations found in drinking water in several developing countries (Ghosh,
398 2007); our low arsenic doses of 0.5 and 1 μM correspond to 64 and 129 ppb, respectively. In
399 the present study, lower doses administered for the short period of six days did not affect
400 testicular development. These results cannot, however, exclude the possibility that chronic
401 long-term exposure to such concentrations of arsenic may be detrimental to the prepubertal
402 testis, and further longer term studies are advisable. Several studies on rodents have revealed
403 arsenic accumulation in the testis and other accessory sex organs (Dua et al., 2015; Pant et al.,
404 2001; Prathima et al., 2018). Such persistent nature after accumulation in the reproductive
405 organs can also adversely impact the developing testis in both human and animals.
406 Furthermore, it is possible that other physiological processes have been compromised outside
407 those assessed in the present study that might influence fertility in the adulthood. In the high
408 arsenic doses experiment here, the lowest concentration of 10 μM, corresponding to 1299 ppb
409 arsenic, was highly detrimental to normal testicular physiology even after six days of exposure,
410 with increased cell death and a reduction in spermatogonial germ cell density. Such dose is
411 below the top concentrations of arsenic found in irrigation shallow tube wells, wells that are
412 used as a source of drinking water for local populations in rural areas of developing countries.

Data here suggest a potential concerning scenario for the population living in the highly arsenic contaminated rural areas. Caution needs to be exercised before extrapolating data obtained from an *in vitro* mouse study such as this to the situation in human and animal populations, in light of the limitations previously discussed: as such, further studies are required in order to confirm the translational risk of future fertility impairment for young boys. This is likely to include the need for *in vivo* studies, in order to fully evaluate the long-term impact, and the effect of metabolic processes on arsenic circulating levels and toxicity. Comparative *in vivo* studies would also be required to validate the *in vitro* system. Such investigation should be extended to the fertility of large animals; in areas where animal husbandry is an important economic factor, arsenic-induced infertility of farm animals could represent a further financial burden for the unstable economy of farmers in developing countries.

In conclusion, using a short term culture system of mouse prepubertal testis, this study shows that exposure to high levels of arsenic for six days caused a dose dependent reduction of spermatogonial germ cells and a marked increase in testicular cell apoptosis.

Conflict of Interest

The authors declare no conflict of interest.

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